Biochemical and Molecular Studies on Differential Production of Piperine and its Precursors in Different Parts of *Piper longum* L.

A Synopsis of the proposed work for
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Mrinalini Prasad

Prof. Prem Kumar Dantu
Supervisor

Prof. D. S. Rao
Head, Department of Botany

Prof. L.D. Khemani
Dean, Faculty of Science

Department of Botany
Dayalbagh Educational Institute
(Deemed University)
Dayalbagh, Agra
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INTRODUCTION

Plants have been the source of medicines since thousands of years as they synthesize a wide variety of chemical compounds that are used to perform important biological functions as pollination attractors, defend against various pests and pathogens and also from herbivorous mammals. At least 12,000 such compounds have been isolated so far; a number estimated to be less than 10% of the total (Tapsell *et al.*, 2006; Lai and Roy, 2004). The World Health Organization (WHO) estimates that 80 percent of the population of some Asian and African countries presently uses herbal medicine for some aspect of primary health care.

Piper longum L. (Piperaceae) commonly known as long pepper or pippali is native of the Indo-Malaya region. It occurs in the hotter and humid parts of India, from Central Himalayas to Assam, Khasi and the Mikir hill, the lower hills of West Bengal, and the evergreen forests of the Western Ghats from Konkan to Travancore and has also been recorded in the Car Nicobar Islands (Manoj *et al.*, 2004).

Long pepper is a slender, aromatic, perennial climber, with large woody roots and numerous creeping, jointed stems with thickened node bearing cordate leaves (Mishra, 2010). The male and female spikes are produced on different plants. The inflorescence is a cylindrical, pedunculate and flowers grow on solitary spike. The female spike is up to 2.5 cm long and 4-5 mm in diameter but the male spikes is larger, slender and narrow. The fruits are small, ovoid berries, shiny blackish green, embedded in fleshy spikes (Zaveri *et al*., 2010).

Secondary plant metabolites are used in signaling and regulation of primary metabolic pathways. Secondary metabolites of plant are commercially important and find use in a number of pharmaceutical compounds (Singh M *et al*., 2012). The fruit of *Piper longum* contains a large number of alkaloids and related compounds, the most abundant of which is piperine, together with methyl piperine, piperonaline, piperettine, asarinine, pellitorine, piperundecalidine, piperlongumine, piperlonguminine, refractomide A,

pregumidiene, brachystamide, brachystamide-A, brachystine, pipercide, piperderidine, longamide and tetrahydropiperine, terahydro piperlongumine, dehydropipernonaline piperidine, piperine, terahydropiperlongumine and trimethoxy cinnamoyl-piperidine and piperlongumine have been found in the root of *P. longum* (Zaveri *et al*., 2010). Piperidine is used as a marker compound in *P. longum* (Saraf and Saraf, 2013). Different plant parts of *P. longum* revealed the presence of various important bioactive compounds viz. alkaloid, flavonoids, glycosides, tannin, phenol and sterol (Rami *et al.*, 2013).

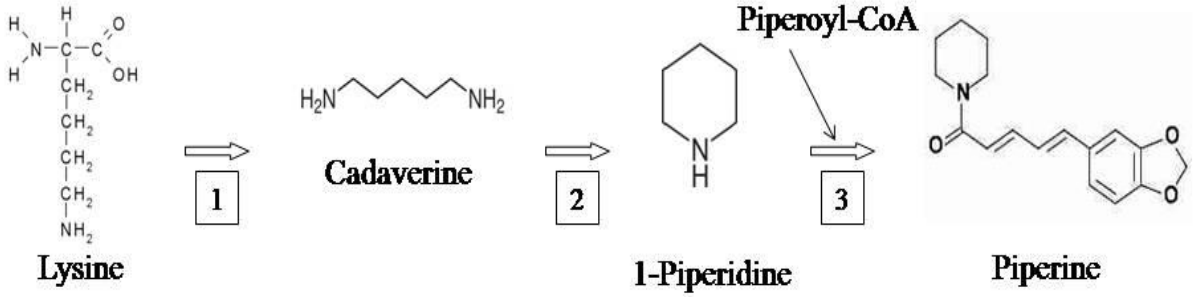
Indian long pepper is a powerful stimulant for both the digestive and the respiratory systems and has been shown to have a rejuvenating effect on the lungs (Charaka referred by Sharma, 1996). Pippali plays an important role in the increased thyroid hormone level and has a thermogenic response, i.e. the release of metabolic heat energy. Several biological activities like immunostimulatory, anti-ulcer (Sharma *et al*., 2012), anti-amoebic, anti-oxidant, hepatoprotective, cardiovascular and anti-inflammatory activities (Chauhan *et al.,* 2011) were reported from the fruit of this plant (Warrier *et al.*, 1995; Dahanukar and Karandikar, 1984). Active biomolecules from *P. longum* proved to be a source to develop MDR (multiple drugs resistant) reversal agents of natural origin to contain the development and spread of plasmid borne multiple antibiotic resistances (Kumar *et al.*, 2013).

Effect of *Piper longum* includes analgesic and diuretic effects, relaxation of muscles tension and alleviation of anxiety (Sunila and Kuttan, 2004). The extract of the root of *Piper* and its major compound, piperine exert anti-oxidant activity and are protective in the myocardial ischemic condition (Joshi *et al.*, 2013). Piperine is extensively used for enhancing the bioavailability and bioefficacy of various drugs (Atal and Bedi, 2010).

Piper longum is rarely cultivated. It is still extensively collected from the wild, threatening the very existence of the plant (Nair, 2000). There is an urgent need not only to cultivate this plant but also develop conservation strategies for it. However, a major impediment to this is the non-availability of large number of propagules as the plant is a

poor seed setter and vegetative propagation is slow. Development of a tissue culture protocol for mass proliferation and conservation could be a viable alternative. Regeneration protocols for this plant have been reported earlier from shoot tips, root, leaf, node and internodes (Soniya and Das, 2002; Rani and Dantu, 2012).

Biosynthesis of piperine has been shown to follow the following pathway involving three steps (http://www.genome.jp/kegg/)



Enzymes required at step 1, 2 and 3 have been identified as L-Lysine decarboxylase (4.1.1.18), Cadaverine aminotransferase and piperidine piperoyl transferase (2.3.1.145), respectively (http://www.genome.jp/kegg/). Condensation of piperidine with piperoyl-CoA to produce piperine was determined in *P. nigrum* (Geisler and Gross, 1990). The biosynthetic pathway from lysine to piperidine through cadaverine was studied in *Pseudomonas aeruginosa* (Fothergill and Guest, 1977). Survey of the literature indicates that not much attention has been paid to the spatio-temporal distribution of piperine and its precursors in *P. longum.*

OBJECTIVES OF PRESESNT STUDY

Spikes and roots of *P. longum* are extensively collected for pharmaceutical uses. These are the reasons of the plant becoming threatened in the wild. The distribution of piperine in various parts of the plant in different seasons and elucidation of the possible reasons of elevated levels of piperine, if at all, in spikes will go a long way in mitigating the threat to the plant. In view of this the present work on *P. longum* has been taken with the following objectives:

1. Estimation of piperine and its precursors in different parts of the plant at different developmental stage and seasons.
2. Preparation of subtraction cDNA library of spikes and leaf.
3. ESTs generation and analysis by using various bioinformatics tool.

REVIEW OF LITERATURE

A large number of compounds now referred to as piperine-type alkaloids or alkamides or piperamides have been isolated commonly from species belonging to the genus piper (piperaceae). The biogenesis of the amides has been investigated and a number of synthetic pathways have been developed to make them readily available for biological studies. The piperine and its analogues are potential pesticides and possess a number of medicinal properties (Okwute and Egharevba, 2013).

The chemistry of members of the family Piperaceae is of great interest owing to the variety of biological properties displayed. A survey of structural diversity and bioactivity reveals that groups of species specialize in the production of amides, phenylpropanoids, lignans and neolignans, benzoic acids and chromenes, alkaloids, polyketides, and a plethora of compounds of mixed biosynthetic origin (Kato and Furlan, 2007).

Piperine was confirmed by qualitative analysis and TLC in *Piper longum*. The purity of the compound was ascertained by UV, FTIR studies and by differential scanning calorimetry (DSC). The anti-mycobacterial activity of the isolated piperine was tested against M. tuberculosis H37Rv strains with standard of Rifampicin by Resazurin Microplate Assay (REMA) (Swapna *et al.,* 2012)

A method was developed for the isolation of piperine from the fruit of *Pipre nigum* (Kanaki *et al.,* 2008). Piperine is present in fruits of *Piper nigrum* and *Piper longum* used as bioavailability enhancer. It was isolated using column chromatography. Characterization of compound was done by spectroscopic technique. A simple, rapid, accurate and specific HPTLC method developed and validated. The method proposed can be used for the routine analysis of both *P. nigrum* and *P. longum* fruit material and its formulations (Hamrapurkar *et al.*, 2011). The n-hexane extracts of 19 Piper species were screened for their 5-lipoxygenase (5-LOX) and cyclooxygenase-1 (COX-1) inhibitory potential. Many of them showed considerable inhibitory activity especially against COX-1 (Stohr *et al.*, 2001).

A bioassay-guided isolation of the ethanol extract from the fruits of *Piper longum* yielded a known piperidine alkaloid, piperine, as a monoamine oxidase (MAO) inhibitor. The inhibition by piperine was found to be reversible by dialysis of the incubation mixture. In addition, the immobility times in the tail suspension test were significantly reduced by piperine, similar to that of the reference antidepressant fluoxetine, without accompanying changes in ambulation when assessed in an open-field. These results suggest that piperine possesses potent antidepressant-like properties that are mediated in part through the inhibition of MAO activity (Ro *et al.,* 2005).

Expression of monoterpenoid indole alkaloids (MIA) pathways are coupled to secretory mechanisms that keep catharanthine and vindoline separated from each other in living plants. Although the entire production of catharanthine and vindoline occurs in young developing leaves, catharanthine accumulates in leaf wax exudates of leaves; whereas vindoline is found within leaf cells (Roepke *et al.,* 2010). The anti-inflammatory activity of two varieties of *Pippali* in acute and sub-acute experimental models of inflammation had been studied in albino rats. It was found that *Chhoti* variety of *Pippali* suppressed inflammation of both acute and sub acute phase, while *Badi* variety of *Pippali* only of acute phase (Kumari *et al.,* 2012).

The phytochemistry of the genus *Piper* is rich in terms of numbers of compounds discovered, but given the diversity of this genus and the intra specific diversity of secondary metabolites in those species that have been examined (Dyer *et al.,* 2004). From the root of *P. longum* L. two alkaloids, piperlongumine and piperlonguminine have been isolated. Piperlongumine and piperlonguminine are shown to be N-(3, 4, 5-trimethoxycinnamoyl) -Δ^5-piperidin-2-one and isobutylamide of piperic acid respectively (Chatterjee and Dutta, 1967). A new acyltransferase from shoots of *Piper nigrum* was detected which catalyses the synthesis of piperine in the presence of piperoyl-coenzyme A and piperidine (Geisler and Gross, 1990).

Piperine (1 - piperoyl piperidine) is the major alkaloid responsible for the pungent smell of the West African pepper, *Piper guineense*. To develop a RP-HPLC method to

standardize the various parts of *P. guineense* using isolated piperine as a biomarker and a secondary reference. The isolated piperine was characterized by carrying out NMR, UV and mass spectroscopy analyses (Adosraku *et al.*, 2013). To established libraries by HPLC chromatograms, where such sample tracing can be compared with reference as fingerprints in the evaluation of authentication and standardization methods (Menghani *et al.*, 2010**).**

Stevia rebuadiana (Bertoni) is known to yield diterpenoid steviol glycosides (SGs), which are about 300 times sweeter than sugar. Expression of various genes of the SGs biosynthesis pathway in different organs of the plant in relation to the SGs content was analyzed. The genes responded to the modulators of the terpenopids biosynthesis (Kumar *et al.*, 2011).

A new application of the carborundum abrasion (CA) technique was developed to obtain epidermis-enriched leaf extracts of *Catharanthus* leaves that can be used to measure alkaloid metabolite levels, enzyme activities and gene expression. It shows that leaf epidermal cells are biosynthetically competent to produce tryptamine and secologanin precursors that are converted via many enzymatic transformations to make 16-methoxytabersonine (Murata and Luca, 2005). The leaf and root transcriptomes of *Catharanthus roseus* were comparatively analyses using two different approaches: (i) indirectly through construction and characterization of separate cDNA libraries; and (ii) directly through a strategically designed suppression subtractive hybridization, using the leaf and root cDNA populations as tester and driver, respectively (Shukla *et al.*, 2006).

Functional genomics approaches, which use combined computational and expression-based analyses of large amounts of sequence information, are emerging as powerful tools to accelerate the comprehensive understanding of cellular metabolism in specialized tissues and whole organisms. As part of an ongoing effort to identify genes of essential oil (monoterpene) biosynthesis, we have obtained sequence information from 1,316 randomly selected cDNA clones, or expressed sequence tags (ESTs), from a peppermint (Mentha x piperita) oil gland secretory cell cDNA library. After bioinformatic selection, candidate genes putatively involved in essential oil biosynthesis and secretion

have been subcloned into suitable expression vectors for functional evaluation in *Escherichia coli* (Lange *et al.*, 2000).

Carborundum abrasion technique was used to extract leaf epidermis–enriched mRNA, thus sampling the epidermome, or complement, of proteins expressed in the leaf epidermis. Random sequencing of the derived cDNA library established ESTs, composed of clusters and singletons. Virtually all known MIA (monoterpenoid indole alkaloids) pathway genes were found in this remarkable set of ESTs, while only four known genes were found in the publicly available *Catharanthus* EST data set. The pathways for triterpene biosynthesis were also identified, and metabolite analysis showed that oleanane-type triterpenes were localized exclusively to the cuticular wax layer. The pathways for flavonoid and very-long-chain fatty acid biosynthesis were also located in this cell type (Murata *et al.*, 2008).

Pseudomonas aeruginosa PACI grows poorly on L-lysine as sole source of carbon. The existence of a route for L-lysine catabolism which differed from those reported previously in other species of *Pseudomonas.* The postulated route, the cadaverine or decarboxylase pathway, is initiated by the decarboxylation of L-lysine and involves the following steps: L-lysine leads to cadverine leads to I-piperideine leads 5-aminovalerate leads to glutarate semialdehyde leads glutarate (Fothergill and Guest, 1977).

PROPOSED METHODOLOGY

- Estimation of piperine and its precursors in different parts of plant at different developmental stages and seasons.
    - Different parts – leaf, nodes, internodes, petiole and spikes.
    - Vegetative stage, Flowering stage and Fruiting stage in different seasons.
- Preparation of subtraction cDNA library of spikes and leaf of *Piper longum*.
    - Collection of leaf and spikes
    - Isolation of total RNA
    - Purification of mRNA
    - Construction of cDNA library
    - Subtraction of cDNA library
- ESTs (Expressed Sequence Tags) generation and analysis by using various bioinformatics tool.
    - cDNA library sequence
    - EST generation and subjected to bioinformatics tool

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